THE UTILIZATION OF CERTAIN HYDROCARBONS BY MICROÖRGANISMS[1]

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INTRODUCTION

Hydrocarbons, as a class, represent compounds with an excellent store of energy. Some idea of the available energy may be obtained by comparing the heat of combustion of some six carbon compounds. For example, glucose yields 674 Calories, as compared to 1,002 Calories from hexane. Knowledge of the biological utilization of hydrocarbons as sources of energy and carbon is fragmentary. However, various investigators have proved that a biological oxidation of these compounds can occur.

Since bacterial decomposition of hydrocarbons has been established, the fact is of significance in considering the carbon cycle. The percentage of carbon in hydrocarbons varies from 80 to 89 per cent; therefore, a significant amount of the world's carbon is combined in this form. According to Egloff (1940) it is becoming more firmly established that perhaps the formation of petroleum is still going on today.

The rôle of bacteria in the production of hydrocarbons is now widely accepted, although, according to Thayer (1931), there is no direct evidence that hydrocarbons of higher molecular weight than methane can be produced by their activity.

REVIEW OF LITERATURE

The first study of the utilization of hydrocarbons by organisms was by Söhngen (1906) and Kaserer (1906). Söhngen isolated

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what he called a methane bacillus which was used by Orla-Jensen (1909) as the type culture for the genus *Methanomonas*, and renamed as *Methanomonas methanica*. (Described in Bergey (1939)). Münz (1920) isolated and studied a somewhat similar methane-oxidizing organism which he classed as a facultative autotrope.

Störmer (1908) isolated an organism which he called *Bacillus hexcarbovorum* that was able to utilize toluene, xylol, and illuminating gas, in addition to methane, as a source of carbon.

Organisms resembling *Bacterium fluorescens*, as described by Lehmann and Neuman (1896), capable of oxidizing methane completely to carbon dioxide and water, were isolated from rice swamp soils by Aiyer (1920).

Söhngen (1913) reported that gasoline, kerosene, paraffin oil, and paraffin wax could be oxidized to carbon dioxide, water, and traces of organic acids by forms isolated from garden soil, ditch water and compost. The organisms studied by Söhngen belonged principally to the genera *Mycobacterium* and *Pseudomonas*. The following organisms were described and studied in this connection: *Mycobacterium phlei*, *M. lacticola*, *M. album*, *M. luteum*, *M. rubrum*, *M. hyolinum*, *Bacterium fluorescens (liquefaciens)*, *B. fluorescens (non-liquefaciens)*, *B. pyocyaneum*, *B. punctatum*, *B. lipolyticum*, and *Micrococcus paraffinae*. Of special interest was the utilization of paraffin wax by members of the genus *Mycobacterium*.

Tausz and Peter (1919) described three new hydrocarbon-utilizing bacteria which they isolated from garden soil and to which the following names were assigned: *Bacterium aliphaticum*, *Bacterium aliphaticum (liquefaciens)* and "Paraffin bacterium." The hydrocarbons used in the enrichment cultures were hexane, cyclohexane and dimethycyclohexane, and paraffin oil respectively. From the accompanying description the first two species were probably members of the genus *Pseudomonas*. They were inert towards cyclic hydrocarbons and hexylene, but attacked paraffinic compounds such as n-hexane, n-octane, di-methyl-octane, n-hexadecane, tricontane, tetratricontane, and other olefinic compounds such as n-caprylene and hexadecylene. The "Paraffin bacterium" was a large gram-positive spore-bearing

rod which liquefied gelatin. It was without effect upon naphthenes, benzoid hydrocarbons, and some paraffins (such as n-hexane and n-octane), but attacked higher paraffins (such as hexadecane, tricontane, and tetratricontane).

Advantage was taken by Tausz and Peter of the fact that these organisms would ferment aliphatic and not cyclic hydrocarbons in removing the former and purifying the latter in mixtures of the two. They also devised a qualitative test for aliphatic hydrocarbons on this basis, since the presence of very small portions of aliphatic compounds in natural oils or artificial mixtures resulted in clouding, due to bacterial growth.

Matthews (1924) observed an increase in the total counts on soil treated with benzene, naphthalene, toluene, phenol, xylene, hexane, pseudocumene, mesitylene, cymene, and pinene, correlated with increases in molecular weights and heat of combustion of the compounds. Her work was continued by Gray and Thornton (1928) who isolated various organisms capable of decomposing aromatic compounds such as naphthalene, toluene, cresol and phenol. Organisms belonging to the following genera were described as capable of utilizing one or more of the compounds studied: *Micrococcus, Mycobacterium, Bacterium, Bacillus* and *Spirillum.* They reported one micrococcus, three mycobacteria, two of the genus bacterium, six pseudomonads, and two vibrios as capable of utilizing naphthalene when grown on a mineral-salts medium.

A paraffin-wax-mineral-salt solution for the isolation of saprophytic members of the genus *Mycobacterium* was used successfully by Büttner (1926) and advocated by him as an enrichment medium. He isolated 12 strains of the mycobacteria, most of which resembled *M. phlei,* and found that several species of the actinomycetes were also capable of growing in such a medium.

Haag (1927) noted that the utilization of commercial paraffin wax by mycobacteria was correlated with the iodine number, indicating that the organisms attack the unsaturated bonds in the molecules. Haag (1927) found that mycobacteria could utilize paraffin while corynebacteria could not and used this as a basis for separating the two.

Jensen (1934) studied saprophytic mycobacteria (including

the avian tubercle bacillus) and corynebacteria and confirmed Haag's work. He found that some species of the mycobacteria such as *M. lacticola*, would use paraffin, but that corynebacteria, such as *C. helvolum* and *C. simplex*, could not utilize it.

Oil-bearing regions, such as the Baku oil field of Russia, were found by Tauson (1929) to contain a great variety of microorganisms which utilized hydrocarbons. He isolated three species of bacteria, *Bacterium naphthalinicus*, *B. naphthalinicus* (*liquefaciens*), and *B. naphthalinicus* (*non-liquefaciens*), which could utilize naphthalene; one species, *B. phenanthrenicus*, which could easily attack phenanthrene; and one species, *B. benzoli*, capable of utilizing benzene, toluene, and xylene. He also studied the oxidation of benzene hydrocarbon derivatives by an organism which he designated as *B. toluolicum*.

Lipman and Greenberg (1932) isolated a coccus, or coccobacillus, from petroleum obtained at a depth of 8,700 feet which was capable of completely oxidizing petroleum to carbon dioxide.

Tauson and Schapiro (1934) observed an increase in the refractive index and saponification numbers with a decrease in the iodine number of lubricating oils as bacterial action progressed.

Harper (1939) noted an increase in soil fertility resulting from leaking natural gas mains and believed that the increased nitrogen content of soils, permeated by natural gas, was due to nitrogen fixation by various clostridia in the soil which utilized the gas as a source of energy in nitrogen fixation.

Organisms capable of decomposing kerosene into methane and possibly ethane were isolated by Thaysen (1940) from the water at the bottom of a kerosene storage tank which had ignited spontaneously. He suggested that organisms could have produced gaseous hydrocarbons which would form an explosive mixture when mixed with the proper amount of air.

Microörganisms capable of attacking petroleum products were obtained from soil by Stone, White, and Fenske (1940) by means of a medium containing mineral salts and petroleum. They found that the oils high in paraffinic hydrocarbons were more readily assimilated than those containing a high percentage

of aromatic compounds. The naphthenic fractions occupied an intermediate position. The organisms were not described but certain recorded characteristics would indicate that they probably belonged to the genus *Pseudomonas*. The following investigators have recorded the utilization of solid paraffin by fungi: Rahn (1906), Söhngen (1913), Gainey (1917), Büttner (1926), and Haag (1926).

CULTURE METHODS

The most successful method of isolating organisms capable of utilizing hydrocarbons is the use of a mineral-salts-hydrocarbon enrichment medium in which the hydrocarbon is the only source of carbon and energy for bacterial growth. This technique was used, with minor modifications, by Söhngen (1913), Tausz and Peters (1919), Tauson (1929), Büttner (1926), Haag (1926), Jensen (1934), and Gray and Thornton (1928).

The following basic salts medium proved to be quite satisfactory:

Water, distilled	1000.0 ml.
$MgSO_4$	0.2 gram
$CaCl_2$	0.02 gram
KH_2PO_4	1.0 gram
K_2HPO_4	1.0 gram
NH_4NO_3 or $(NH_4)_2SO_4$	1.0 gram
$FeCl_3$	2 drops conc. sol.

The medium is adjusted to pH 7.0 to 7.2 with dilute NaOH. Two per cent of washed agar is added whenever a solid medium is needed.[2] In some instances the source material was plated on nutrient agar and selected colonies were later tested on the mineral-salts medium containing only the hydrocarbon as source of carbon. In most instances mineral-salts agar plates were streaked with source material and isolations made therefrom. If volatile hydrocarbons, such as gasoline, were used, the petri dishes were inverted and the hydrocarbon poured into the lid. The volatile hydrocarbon vapors were sufficient to support bac-

[2] Referred to in this paper as a "mineral-salts medium."

terial growth. If relatively non-volatile hydrocarbons were used, such as kerosene and light oils, the material was poured over the surface of the inoculated agar, a procedure that did not interfere with the growth of the cultures.

For liquid cultures the hydrocarbon was added to the mineral-salts solution. If solid paraffin was to be added, both the salts medium and paraffin were sterilized separately, and the paraffin added to the medium while in a melted condition. This tends to give a rough, irregular mass of paraffin, which offers sufficient surface for bacterial action. Liquid hydrocarbons, such as kerosene, were layered on the surface of the medium.

Only small quantities of hydrocarbon material were necessary, approximately 5 per cent being most commonly used. However, if a light, volatile hydrocarbon such as gasoline were used, thick layers did not affect the growth. Liquid hydrocarbons, with the exception of viscous oils, do not retard the diffusion of oxygen sufficiently to prevent the growth of aerobic cultures.

TABLE 1

Effect of pH on hydrocarbon utilizing cultures

	pH RANGE							
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
"Culture X"	+	++	+++	+++	+++	+++	++	+
Ps. #8	+	+++	++++	+++	+++	+++	++	+
P. pyocyaneus	++	+++	++++	++++	+++	+++	+++	++
P. aeruginosa	++	+++	++++	+++	+++	+++	+++	+

The influence of the hydrogen-ion concentration of the medium was determined by streaking a series of mineral-salts-agar plates, ranging in pH from 6.0 to 9.0, with various cultures.

The results recorded in table 1 indicate that the organisms were not extremely sensitive to changes in hydrogen-ion concentration, since they grew well in media that had a pH range of 6.5 to 9.0. This is not in harmony with Tauson's (1929) findings with a member of the *Pseudomonas* group in which he found that the medium must be approximately neutral.

Nature of hydrocarbons used in this study

"Skelly-solve" (B) is a commercial product used in various laboratories as a solvent for extraction purposes. It is a mixture containing low b. p. hydrocarbons such as the hexanes. This product was not further purified for this work.

The gasoline used was Sinclair H-C Regular further purified by treating with sulfuric acid, washing several times, treating with sodium hydroxide and redistilling. This treatment should remove all unsaturated hydrocarbons and organic acids that might be present.

The kerosene designated as raw kerosene was of the ordinary commercial grade, while that designated as treated kerosene had been treated several times with concentrated H_2SO_4, neutralized, and then redistilled.

The light and heavy mineral oils were oils sold by Parke-Davis and Company for medicinal purposes.

The paraffin wax was a commercial embedding product with a melting point of 52°C.

The hydrocarbons such as raw kerosene, mineral oils and paraffin wax were sterilized by autoclaving at seventeen pounds pressure for one hour. Gasoline and treated kerosene were not sterilized but were caught in sterile bottles on redistillation.

Sources of cultures used in this study

Materials normally in contact with hydrocarbons would seem to offer the greatest possibilities of isolating cultures of microorganisms capable of utilizing hydrocarbons. Accordingly attempts were made to isolate hydrocarbon-utilizing organisms from material taken from oil wells, sedimentation ponds, and water commonly found in the bottom of large storage tanks.

Several species of *Pseudomonas* were isolated from water taken from the bottom of storage tanks containing products such as finished, re-run cracked gasoline, finished gasoline, and finished kerosene. One sample of water from the bottom of a distillate tank was found to give a plate count of 981,000 bacteria per ml. A culture isolated from this source was found to be a "slow-

lactose fermenting" organism possessing marked saccharolytic action on sucrose, galactose, mannitol, maltose, and glucose. This organism, hereafter designated as "Culture X," is unique in that it can utilize the hydrocarbons found in "Skelly-solve," gasoline, and kerosene, but it does not seem to be able to utilize mineral oils and paraffin wax very readily.

Crude oil samples from an old well, the production of which was declining do to the infiltration of subterranean water, yielded a culture of *Pseudomonas*.

A sample taken from a sedimentation pond containing an emulsion of crude oil and water yielded numerous small, discrete, dull-red colonies of a large micrococcus on the mineral salts agar plates covered with kerosene. When cultured on nutrient agar they were found to be banded rods, later identified as a species of *Corynebacterium*. Another of this genus was isolated from oil-saturated soil from the vicinity of an oil well.

As a result of the present work with certain species of the corynebacterium group, some results contradictory to those of Haag (1926) and Jensen (1934) were obtained. In addition to cultures of *Corynebacterium simplex*, *Corynebacterium fimi*, and *Corynebacterium tumescens*, four unidentified species of *Corynebacterium* and one species of the genus *Proactinomyces* secured from Dr. F. E. Clark, Department of Microbiology, United States Department of Agriculture, were studied. *Corynebacterium simplex* and three of the unidentified corynebacteria grew quite well in the mineral-salts medium to which solid paraffin (M.P. 52°), or light oil had been added. In addition *Corynebacterium simplex* grew on kerosene. The cultures *Corynebacterium fimi* and *Corynebacterium tumescens* would not grow, thus confirming Jensen's (1934) work in regard to these two cultures.

The work of various investigators, including our own, indicates that the *Pseudomonas* type of organisms plays an important rôle as a hydrocarbon-utilizing group of bacteria. In order to study the group more in detail, stock cultures of *Pseudomonas* from various other institutions and the American Type Culture Collection were placed on mineral-salts agar which contained kerosene as the sole source of carbon and energy. The relative growth of each of these cultures is indicated in table 2.

The results indicate that the ability to utilize hydrocarbons is a common characteristic of this genus, particularly since known

TABLE 2

Growth of stock cultures of Pseudomonas on mineral-salts agar with kerosene

NAME OF CULTURE	ORIGIN AND HISTORY	RELATIVE GROWTH
P. aeruginosa	A. T. C. C.—256	+
P. aeruginosa	A. T. C. C.—262	+++
P. aeruginosa	A. T. C. C.—914	++++
P. aeruginosa	U. S. D. A. Food and Drug—100	++
P. aeruginosa	U. S. D. A. Food and Drug (sour cream)—101	+
P. aeruginosa	U. S. D. A.—Coon (bovine mastitis)—A118	0
P. aeruginosa	U. S. D. A.—Coon (bovine mastitis)—1441	++
P. aeruginosa	U. S. D. A.—Coon (bovine mastitis)—1445	++
P. aeruginosa	University of Illinois—208	++++
P. pyocyaneus	Cornell University (Burnett)—54	+++
P. pyocyaneus	Cornell University (chicken)—55	++
P. pyocyaneus	Cornell University (turkey)—56	+++
P. pyocyaneus	Cornell University (swine)—57	+++
P. pyocyaneus	Cornell University (water)—58	++++
P. pyocyaneus	Yale University—C9	++++
P. pyocyaneus	Yale University—C10	+++
P. pyocyaneus	Yale University (Brigham)—C11	+++
P. pyocyaneus	University of Colorado no. 10628	++++
P. pyocyaneus	University of Colorado no. 11257	+++
P. pyocyaneus	University of Colorado no. 11368V	+++
P. (species unknown)	Oklahoma A. and M. (water) (B)	0
P. (species unknown)	Oklahoma A. and M. (water) (C)	+++
P. (species unknown)	Harnden* (Hotis)	+++
P. (species unknown)	Harnden (Tiny)	+++
P. (species unknown)	Harnden (pig spleen)	++
P. (species unknown)	Harnden (raw milk)	+++
P. (species unknown)	Harnden (bovine mastitis)	+++
P. (species unknown)	Harnden (abscess, horse)	++
P. (species unknown)	Harnden (bear feces)	++++
P. (species unknown)	Harnden (ice cream)	++
P. (species unknown)	Harnden (racoon feces)	++

+, slight growth; ++, moderate growth; +++, good growth; ++++, excellent growth.

* These cultures were isolated by Dr. E. E. Harnden of Oklahoma A. and M. College.

cultures, such as the type species *Pseudomonas aeruginosa*, were found to possess this ability.

Stock cultures of *Mycobacterium phlei, Mycobacterium leprae* and *Mycobacterium smegmatis* were also able to grow quite well in mineral salts medium to which mineral oil or paraffin wax had been added. Thirty-eight stock cultures of the *Proteus* group, including *Proteus vulgaris,* did not grow under these conditions.

Other miscellaneous stock cultures which were tried on the mineral-salts agar with kerosene, but which did not show growth were: *Mycoplana bullata, Mycoplana dimorpha, Escherichia coli, Staphylococcus aureus, Azotobacter chroococcum, Serratia marcescens, Aerobacter aerogenes, Rhizobium trifolium, Lactobacillus casei, Bacillus subtilis, Bacillus mycoides, Bacillus mesentericus, Serratia marcescens, Sarcina lutea, Spirillum ribrum, Eberthella typhosa, Shigella dysenteriae, S. gallinarum, Salmonella paratyphi, S. schottmülleri, S. pullorum,* and *S. enteritidis.*

RESULTS OBTAINED

Quantitative bacteriological counts were made by the dilution-plate method with nutrient agar as the culture medium. The cultures were grown in a 125 ml. Erlenmeyer flask containing 50 ml. of mineral-salts medium and 3 ml. of the various hydrocarbon products. Counts were always made on control flasks without hydrocarbons.

Most of the *Pseudomonas* species gave the greatest bacterial counts on petroleum fractions such as kerosene, light and heavy mineral oil, and paraffin wax (see tables 3, 4, 5, and 6, and figs. 1, 2, 3, and 4). These cultures were incubated at room temperature which averaged about 27°C. although there was considerable variation.

"Culture X" utilized the lighter hydrocarbons and kerosene quite readily (see table 5) but not the mineral oils or paraffin wax.[3] This preference might be explained upon the assumption that the organisms could utilize only straight chain molecules (paraffinic). To test this point a series of test tubes containing the mineral-salts medium and various pure hydrocarbon compounds were inoculated with this organism. It was found that

[3] This organism may be similar to the cultures described by Tausz and Peter (1919) which he claimed would attack only paraffinic compounds.

"Skelly-solve," which is a hydrocarbon fraction containing a high percentage of hexane, was readily utilized, but cyclic compounds, such as benzene, toluene, xylene, and light mineral oil, were not utilized. The *Pseudomonas* strains, which could utilize mineral oils quite readily, were also able to utilize certain cyclic com-

TABLE 3

Counts per ml. of Pseudomonas pyocyaneus (no. 58) grown on various petroleum products in flasks

(Initial count: 5,700,000 per ml.)

INCUBATION TIME	CONTROL	GASOLINE	SKELLY-SOLVE	TREATED KEROSENE	RAW KEROSENE	LIGHT MINERAL OIL	HEAVY MINERAL OIL	PARAFFIN WAX
days								
1	5,750	7,750		25,300	20,050	25,250	18,550	
2	18,200	8,500	107	536,500	412,500	610,000	215,000	79,500
5	18,000	9,500	83	2,550,000	1,864,000	1,962,000	890,000	596,000
7	12,670	1,400		2,575,000	2,810,000	1,930,000	2,010,000	1,475,000
9	20,150	1,780	630	2,450,000	1,710,000	3,480,000	2,160,000	3,880,000
11	15,100	45,700	1,640	2,175,000	990,000	3,670,000	2,795,000	2,085,000

TABLE 4

Counts per ml. of Pseudomonas culture (no. 8) on various petroleum products

(Initial count: 4,150,000 per ml.)

INCUBATION TIME	CONTROL	GASOLINE	SKELLY-SOLVE	TREATED KEROSENE	RAW KEROSENE	LIGHT MINERAL OIL	HEAVY MINERAL OIL	PARAFFIN WAX
days								
2	4,900	3,485	168,000	361,500	362,000	207,500	111,500	51,000
4	6,100	1,830	136,500	2,615,000	2,245,000	1,175,000	206,000	745,000
6	5,250	2,400	18,500	2,130,000	1,625,000	1,020,000	223,000	1,270,000
11	5,200	695	127,500	2,625,000	1,635,000	1,070,000	385,000	1,535,000

Three zeros omitted from each number in table.

pounds. Final proof of a preference for aliphatic hydrocarbons by these cultures must await tests with pure naphthenic and aliphatic hydrocarbons.

Corynebacterium simplex could assimilate the same hydrocarbons as the pseudomonas species, but it could not tolerate the light hydrocarbons, such as "Skelly-solve," which acted as a

germicide under the conditions of this experiment (see tables 6 and 7).

Respiration. Haag (1926) reported that the ratio between the carbon dioxide produced and the paraffin used by a culture was equal to 0.37. This ratio held true for various organisms, such as *M. lacticola, M. phlei, P. pyocyaneum,* and *Actinomycetes*

TABLE 5

Counts per ml. of "Culture X" grown on various petroleum products

(Initial count: 2,360,000 per ml.)

INCUBATION TIME	CONTROL	GASOLINE	SKELLYSOLVE	TREATED KEROSENE	RAW KEROSENE	LIGHT MINERAL OIL	HEAVY MINERAL OIL	PARAFFIN WAX
days								
2	14,600	2,970	40,000	203,500	59,000	10,450	17,050	20,000
5	13,400	6,800	1,091,000	235,000	242,000	14,400	13,800	19,150
7	15,750	79,500	145,000	164,500	146,500	12,050	17,650	23,150
9	18,600	162,500	30,000	165,500	147,500	12,450	18,850	25,900

TABLE 6

Counts per ml. of Corynebacterium simplex grown on various petroleum products

(Initial count: 7,150,000 per ml.)

INCUBATION TIME	CONTROL	GASOLINE	SKELLYSOLVE	TREATED KEROSENE	RAW KEROSENE	LIGHT MINERAL OIL	HEAVY MINERAL OIL	PARAFFIN WAX
days								
3		N*	370	35,150	31,400	1,211,000	705,000	N
6	490	R*	N	6,000	9,850	965,000	1,195,000	R
8	418	7,200	N	1,565,000	273,500	2,050,000	1,685,000	65,000
11	500	1,615	N	1,635,000	1,735,000	1,930,000	1,040,000	750,000
14	495	480	N	530,000	1,515,000	1,765,000	925,000	1,390,000

Three zeros omitted from each number in table.

* N = no growth; R = reinoculated.

chromogenes. However, he could recover only about 80 per cent of the theoretical CO_2. The remainder was believed to have been used by the bacteria in the synthesis of protoplasm and other organic compounds, such as fatty acids.

Stone, White, and Fenske (1940) determined the respiratory quotient of bacteria on various petroleum products, such as

neutral oil, and secured a ratio of CO_2/O_2 varying from 0.50 to 0.70. In some preliminary studies with the organisms described in this paper, the respiratory quotients varied from 0.30 to 0.70.

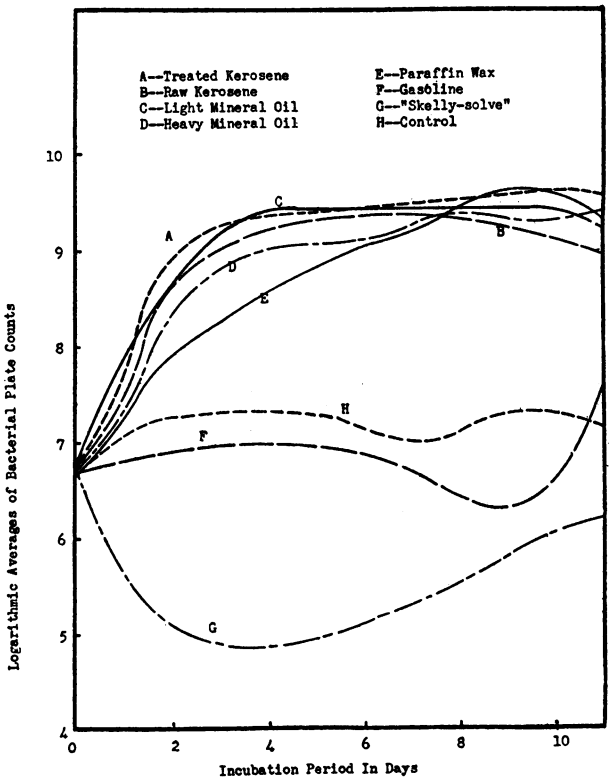

FIG. 1. GROWTH OF PSEUDOMONAS PYOCYANEUS (No. 58) ON VARIOUS PETROLEUM PRODUCTS
See table 3

The utilization of oxygen combined in the form of nitrates and sulfates is governed by experimental conditions. The utilization of these compounds is greater in a respirometer, where the oxygen supply is somewhat limited, than it is in flasks open to the atmosphere. The *Pseudomonas* cultures exhibited a higher demand for sulfates than the other cultures studied. However, combined oxygen in the form of nitrates and sulfates is not essential in the

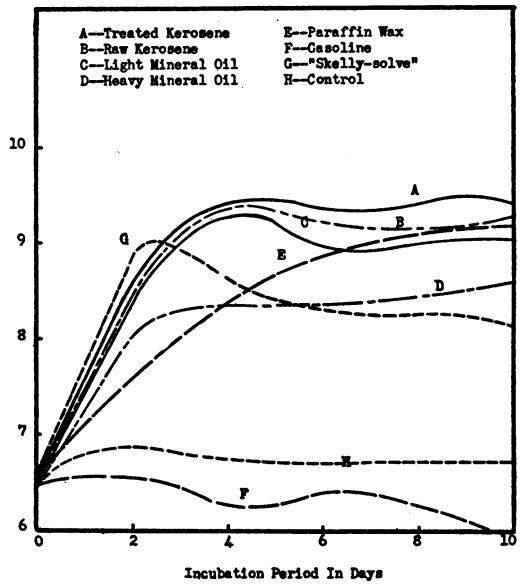

FIG. 2. GROWTH OF PSEUDOMONAS CULTURE NO. 8 ON VARIOUS PETROLEUM PRODUCTS

See table 4

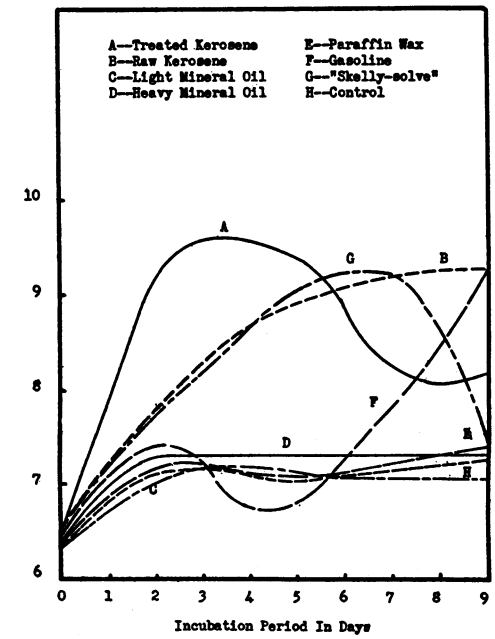

FIG. 3. GROWTH OF CULTURE X ON VARIOUS PETROLEUM PRODUCTS

See table 5

utilization of hydrocarbons by these bacteria. This was demonstrated by arranging a series of tubes of a mineral-salts medium which lacked one or all of the compounds containing oxygen.

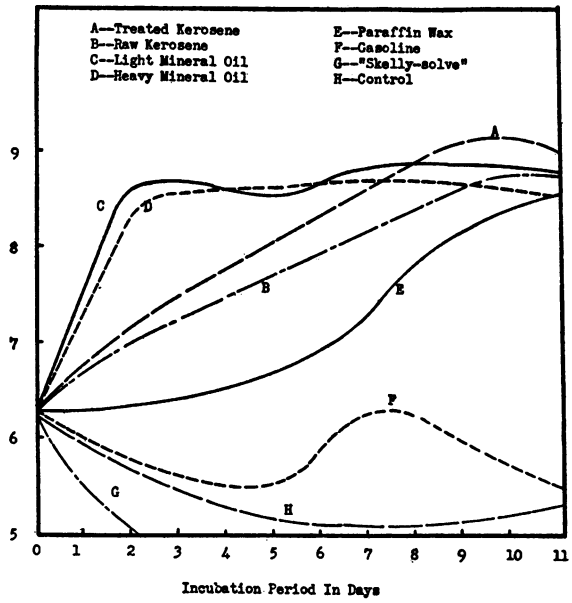

FIG. 4. GROWTH OF CORY. SIMPLEX ON VARIOUS PETROLEUM PRODUCTS
See table 6

TABLE 7

Comparison of the utilization of hydrocarbons by various bacteria

	"CULTURE X"	P. PYOCYANEUS #58	C. SIMPLEX
"Skelly-solve"	++++	++++	0
Light oil	0	+++	+++
Benzene	0	++	0
Toluene	0	++	+++
Xylene	0	+++	++++

++++ = vigorous growth; +++ = moderate growth; + = slight growth.

The following substitutions were made in this medium: ammonium chloride was used in place of ammonium nitrate; potassium sulfide and magnesium chloride were used in place of magnesium sulfate. The concentration of the phosphate ions was reduced

to 50 p.p.m. in the low-phosphate medium and calcium carbonate was added to neutralize any acids that might be formed. A medium which did not contain any combined oxygen was made by employing all of the substitutions mentioned above. Each culture was tried on a series of these tubes containing kerosene and light mineral oil and the relative amount of growth noted (see table 8).

The growth of *Pseudomonas* cultures where hydrocarbons were the only source of energy was dependent upon the concentration

TABLE 8

Effect of combined oxygen on bacterial growth in the presence of air

HYDROCARBON	MEDIUM	CORYNEBACTERIUM SIMPLEX	PROACTINOMYCES SPECIES (17A)	PSEUDOMONAS STRAIN NO. 6	PSEUDOMONAS STRAIN NO. 8	PSEUDOMONAS PYOCYANEUS NO. 58	"CULTURE X"
Light oil.....	Complete	+++	+++	+++	+++	+++	0
Kerosene.....	Complete	0	0	+++	++++	++++	+++
Light oil.....	Minus NO_3	+++	+++	+++	+++	+++	0
Kerosene.....	Minus NO_3	0	0	+++	++++	++++	+++
Light oil.....	Low PO_4*	+++	+++	++	++	++	0
Kerosene.....	Low PO_4*	0	0	+	+	++	+
Light oil.....	Minus SO_4	++++	+++	+++	+++	+++	0
Kerosene.....	Minus SO_4	0	0	+++	+++	++++	+++
Light oil.....	Minus all	+++	+++	0	+++	++	0
Kerosene.....	Minus all	0	0	+++	++++	+++	+++

++++, excellent growth; +++, good growth; ++, moderate growth; +, slight growth.

* It was necessary to add 50 p.p.m. of phosphate to secure minimum growth.

of phosphates. A concentration of 50 p.p.m. of phosphates was only sufficient for minimum growth. The corynebacteria species were not affected by this lack of phosphate. It may be possible that under certain conditions phosphorylation plays as important a rôle in hydrocarbon respiration as it does in carbohydrate respiration.

The preference of the corynebacteria for light mineral oil is quite evident, since good growth was observed in the presence of this compound but not in the presence of kerosene. In this connection it is interesting to recall that "Culture X" utilizes kerosene more easily than it does light mineral oil.

Acid production. The production of organic acids by hydrocarbon-utilizing bacteria was apparently not appreciable, as is seen by studying table 9. This is representative of the changes in pH values produced by various cultures under these conditions. These cultures do not produce appreciable quantities of acid from carbohydrates, so it is not surprising that acids are not produced in abundance from hydrocarbons. However, there is some evidence of acid production. This is revealed in the ease of emulsification of the hydrocarbons following bacterial action. This emulsifying characteristic, due to bacterial action, was noted also by Stone, White, and Fenske (1940). An increase in the saponification number of mineral oil was noted by Tauson and Schapiro (1934). They believed that this was evidence of the presence of fatty and naphthenic acids. The evidence indicates that long-chain acids are formed, but that they are extremely weak; consequently, no great changes in pH are produced.

Other changes produced in certain hydrocarbons by bacterial action. The only previous work pertaining to changes produced in the petroleum fractions as a result of bacterial action was by Tauson and Schapiro (1934). Using mixed cultures they observed that the refractive index, saponification number, and iodine number increased as bacterial action took place. They also state that the organisms first attack the unsaturated hydrocarbons and reduce the iodine number. As the action continues, unsaturated hydrocarbons may again be produced as intermediate products.

The following procedure was used in our determination of the changes produced in kerosene, due to bacterial action:

To each of four one-liter Erlenmeyer flasks were added 300 ml. of mineral-salts medium and 300 ml. of kerosene. Two of the flasks were inoculated with *P. pyocyaneus* (no. 58), and two were left uninoculated as controls. One of each pair was aerated, and the other allowed to remain unagitated. After one week of incubation at room temperature, the flasks were analyzed for organic acids and alcohols, and a distillation run on the kerosene to see if any changes in the fractional boiling points could be detected. The results are recorded in table 10.

If the distillation data of the controls are compared to those of the kerosene which had been acted on by bacteria, no essential

TABLE 9

Change in pH produced by cultures on various hydrocarbons

	IN RESPIROMETERS				IN FLASKS	
	Ps. pyocyaneus no. 58	"Culture X"	*Pseudomonas* strain no. 8	*Cory. simplex*	*Pseudomonas* strain no. 8	*Cory. simplex*
Culture medium alone	6.98	7.10	6.90	6.89		
Control—no hydrocarbon	6.60	N*	6.87	6.85	6.87	6.90
Gasoline	6.66	6.82	N	N	N	N
"Skelly-solve"	N*	6.85	N	N	N	N
Raw kerosene	5.65	6.61	6.57	6.81	5.45	6.19
Treated kerosene	5.81	6.78	6.57	6.65	5.45	5.79
Light oil	5.50	6.88	6.53	6.59	5.92	5.85
Heavy oil	6.52	N	6.70	6.59	6.53	6.26
Paraffin wax	6.15	N	6.69	6.71	6.63	6.33

* N = no growth.

TABLE 10

Distillation tests on kerosene after bacterial action

(*P. pyocyaneus* culture no. 58)

PER CENT DISTILLED	CONTROL (AERATED)	CONTROL (NOT AERATED)	NO. 58 (AERATED)	NO. 58 (NOT AERATED)
	Initial b.p.			
	120°	116°	120°	120°
	b.p.	*b.p.*	*b.p.*	*b.p.*
10	193	190	189	184
20	199	196	195	194
30	202	201	201	202
40	211	205	206	204
50	217	215	213	215
60	223	222	214	221
70	227	228	226	229
80	211	213	232	223
90	225	223	243	243
End point	232	228	257	248

differences will be detected until the point is reached where 80 per cent by volume had been distilled. At this point the sample

which had been subjected to bacterial action showed a marked rise in the boiling point temperature, whereas the controls did not. This average increase of some 22°C. was maintained to the end of the distillation. The increase is thought to be due to the formation of polymers of unsaturated hydrocarbons during the process of distillation. The unsaturation appears to be due to the dehydrogenation of the kerosene by bacterial action. This observation confirms that of Tauson and Schapiro (1934).

SUMMARY AND CONCLUSIONS

Cultures of organisms capable of using petroleum fractions such as "Skelly-solve," gasoline, kerosene, light and heavy mineral oils and paraffin wax as the source of carbon and energy for their metabolism were isolated from various sources. Oil-bearing soil, sedimentation ponds, and "water bottoms" of various petroleum storage tanks, were found to be good sources for the isolation of organisms of this type. Organisms possessing this ability are not necessarily confined to such habitats, since practically all the *Pseudomonas* cultures, regardless of their origin, were capable of utilizing kerosene. This was demonstrated by the fact that cultures isolated from various sources such as abscesses, mastitis, water, and fecal matter of animals, were all able to utilize it.

Bacteria of other genera were also found capable of this activity, including certain species of the micrococci, corynebacteria, and "Culture X." "Culture X" exhibited a preference for paraffinic to the naphthenic or cyclic hydrocarbons. Many of the cultures were able to withstand as high as 10 to 15 transfers under kerosene without diminution in growth, thereby indicating that accessory growth factors are not needed, or that the organisms were able to synthesize these substances.

Respiration studies indicated that the hydrocarbons were oxidized largely to carbon dioxide and water. The respiratory quotients of various bacterial cultures on different hydrocarbons varied from 0.30 to 0.70. No direct correlation between the respiratory quotient and the nature of the hydrocarbon was observed.

Some evidence was obtained to show that long-chain organic acids and unsaturated hydrocarbons were formed during the bacterial decomposition of the hydrocarbon fractions. The formation of organic acids was indicated by small changes in the pH of the medium and by the increased ease of formation of emulsions of oil and water.

The bacterial production of unsaturated hydrocarbons was indicated by changes in the distillation temperatures of the kerosene. The boiling point of the last 20 per cent of kerosene distilled was higher, probably indicating that polymers with a higher boiling point were formed from the unsaturated hydrocarbons during the process of distillation.

As a result of this investigation, it has been established that the bacterial utilization of hydrocarbons is a characteristic common to many types of microörganisms and that in nature this process probably occurs to a greater extent than is generally recognized. The oxidation of hydrocarbons was found to occur on simple media; in fact, ordinary well water at the bottom of a distillate tank was able to support a bacterial count of approximately 900,000 organisms per ml.

The respiration studies indicated that the oxidation of hydrocarbons is very similar to the oxidation of other organic compounds, and that such end products as carbon dioxide, water, organic acids, and unsaturated hydrocarbons are produced.

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